Published in final edited form as:

Am J Transplant. 2016 October ; 16(10): 2842–2853. doi:10.1111/ajt.13821.

Adoptive transfer of tracer alloreactive CD4⁺ TCR-transgenic T cells alters the endogenous immune response to an allograft

Michelle L. Miller¹, Jianjun Chen², Melvin D. Daniels^{2, @}, Matthew G. McKeague¹, Ying Wang¹, Dengping Yin², Vinh Vu², Anita S. Chong^{2, *}, and Maria-Luisa Alegre^{1, #, *}

¹Section of Rheumatology, Department of Medicine, University of Chicago

²Section of Transplantation, Department of Surgery, University of Chicago

Abstract

T cell receptor transgenic (TCR-Tg) T cells are often used as tracer populations of antigen-specific responses to extrapolate findings to endogenous T cells. The extent to which TCR-Tg T cells behave purely as tracer cells or modify the endogenous immune response is not clear. To test the impact of TCR-Tg T cell transfer on endogenous alloimmunity, recipient mice were seeded with CD4⁺ or CD8⁺ TCR-Tg or polyclonal T cells at the time of cardiac allograft transplantation. Only CD4⁺ TCR-Tg T cells accelerated rejection, and unexpectedly led to a dose-dependent decrease in both transferred and endogenous T cells infiltrating the graft. In contrast, recipients of CD4⁺ TCR-Tg cell exhibited enhanced endogenous donor-specific CD8⁺ T-cell activation in the spleen and accelerated alloantibody production. Introduction of CD4⁺ TCR-Tg T cells also perturbed the intra-graft accumulation of innate cell populations. Thus, transferred CD4⁺ TCR-Tg T cells alter many aspects of endogenous alloimmunity, suggesting that caution should be used when interpreting experiments utilizing these adoptively-transferred cells, as the overall nature of allograft rejection may be altered. These results may also have implications for adoptive CD4⁺ T cell immunotherapy in tumor and infectious clinical settings as cell infusion may have additional effects on natural immune responses.

Introduction

In animal studies, T cell receptor transgenic (TCR-Tg) T cells, which are monoclonal T cells specific for a known antigen, are often used as a convenient tool for the study of antigen-specific T cell responses. It is easy to obtain sufficient numbers of antigen-specific T cells from TCR-Tg mice and these cells, once transferred, can be identified by their particular TCR V α or V β subunit, a clonotypic antibody, or a congenic marker. This approach has been used to track the fate and function of antigen-specific T cells including their deletion,

[#]To whom correspondence should be addressed: Maria-Luisa Alegre, M.D., Ph.D., The University of Chicago, Department of Medicine, 924 E. 57th St., JFK-R312, Chicago, IL 60637; tel: 773-834-4317; fax: 773-702-4394; malegre@midway.uchicago.edu.

^{*}Co-last authors

[@]Current address: Department of Biological Sciences, 9501 S. King Drive, Chicago State University, Chicago, IL 60628

Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

proliferation, differentiation, or cytokine production (1,2). Furthermore, this approach with its ability to transfer a set number of cells has allowed estimation of the number of antigen-specific cells required to clear an infection, reject an organ or tumor, or to overcome a therapeutic intervention (1–4). The fate and numbers of the TCR-Tg cells are then often extrapolated to endogenous T cell responses. Importantly, it has been demonstrated in models of infection that TCR-Tg T cells can compete with endogenous T cells specific for the same antigen (5). These observations suggest that the introduced TCR-Tg T cells are not simply recruited into the immune response but they may also in fact alter the immune responses on which they are supposed to report.

In this study, we transferred TCR-Tg and polyclonal T cells into mice receiving fully allogeneic cardiac allografts to investigate their fate and effects on the endogenous alloimmune response. The transfer of 10^4 – 10^5 CD4⁺ TCR-Tg T cells/mouse, a similar dose to what has been frequently used for mechanistic studies of allograft rejection and tolerance, significantly modified the endogenous immune response to the allograft, increasing both CD8 and alloantibody responses, while paradoxically limiting the accumulation of intra-graft T cells and altering the profile of graft-infiltrating innate immune cells. These pleiotropic alterations in the endogenous immune response as a result of CD4⁺ TCR-Tg T cell transfer urge caution for the use of these cells in animal models and patient therapies.

Materials and Methods

Mice

C57BL/6 and BALB/c mice were purchased from Envigo RMS, Inc. (Indianapolis, IN). OT-I Rag-KO, MD4, μ MT^{-/-} and CD45.1 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Ovalbumin-transgenic (OVA-Tg) mice on a BALB/c background, that express membrane OVA under the control of the β -actin promoter, were a generous gift by Elizabeth Ingulli (when at the University of Minnesota). 2W1S-OVA mice were provided by James Moon (Harvard) and crossed once to BALB/c mice to generate F1 mice. TCR75 TCR-Tg mice were obtained from R. Pat Bucy (University of Alabama-Birmingham) and crossed to CD45.1 mice. TEa TCR-Tg mice were obtained from Alexander Rudensky (when at the University of Washington) and crossed to CD45.1 mice. Mice were housed under specific pathogen-free conditions and used in agreement with the University of Chicago's Institutional Animal Care and Use Committee, according to the National Institutes of Health guidelines for animal use.

Heart transplantation

Transplantation of cardiac allografts was performed using a technique adapted from that originally described by Corry et al. (6). Cardiac allografts were transplanted in the abdominal cavity by anastomosing the aorta and pulmonary artery of the graft end-to-side to the recipient's aorta and vena cava, respectively. The day of rejection was defined as the first day of cessation of heartbeat in the graft. Graft rejection was verified in selected cases by necropsy.

Adoptive transfer of cells

Spleen and lymph node cells were isolated from donor mice. Cells were enumerated with an Accuri C6 flow cytometer (BD Biosciences) and a subset of cells were stained for CD4 or CD8, V β or V α , the congenic marker CD45.1 or CD45.2 and CD44. The percentage of CD44^{lo}, congenically marked TCR-Tg T cells was used to calculate the total number of cells for the adoptive transfer. Cells were injected retro-orbitally in 200 μ l of phosphate-buffered saline (PBS) on the day of transplantation unless otherwise indicated.

Isolation of graft-infiltrating cells

Cardiac grafts were rinsed in situ with Hanks balanced salt solution (HBSS) containing 1% heparin. Explanted hearts were cut into small pieces and digested for 40 min at 37°C with 400 U/ml collagenase IV (Sigma), 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, Cellgro) and 0.01% DNase I (MP Biomedicals) in HBSS (Cellgro). Digested suspensions were washed with an equal portion of complete Dulbecco's Modified Eagle medium (DMEM) containing 5% fetal bovine serum, passed through a nylon mesh and centrifuged. Cells were either used for flow cytometry or incubated for 4 h with phorbol myristate acetate (PMA) and ionomycin in the presence of brefeldin A.

Intracellular staining and flow cytometry

Lymphocytes were isolated from spleens and heart grafts and processed into single cell suspensions. Cells were stained first with a fixable live/dead stain (Aqua, Invitrogen) and then with anti-CD4 (L3T4), anti-CD8 (Ly2), anti-CD45.1 (A20), anti-V β 8-3 (1B3.3) or anti-V α 2 (B20.1) and anti-CD45.2 (104), anti-CD11b (M1/70), anti-Gr-1 (RB6-8C5), anti-TCR β (H57-597), anti-CD19 (eBio1D3), anti-CD45 30-F11) mAbs. For interferon gamma (IFN γ) intracellular staining, cells were stimulated with PMA (Sigma), ionomycin (Sigma) and brefeldin A (BD Pharmingen) for 4 h, prior to staining for viability and surface markers. Surface-stained cells were then fixed with the Foxp3 fixation permeabilization buffer kit (eBioscience) for 15 min at room temperature, washed with 1 $\times$ permeabilization buffer, stained using anti-IFN γ (XMG1.2) for 30 min at room temperature, washed again, and analyzed by flow cytometry. For T follicular helper (Tfh) analysis, cells were stained with surface antibodies and CXCR5-bio for 2 hours at 4°C, followed by two washes with FACS buffer and 30 minute staining with streptavidin-Percp-e710 at 4°C. For staining B cells, anti-B220 (RA3-6B2), anti-Fas (15A7) and anti-GL-7 (GL7) were used as well as a dump gate including anti-CD3 (145-2C11), anti-Ter119 (TER119), anti-F4/80 (BM8), anti-CD49b (DX5), and anti-Gr-1 (RB6-8C5). All mAbs were from BD Biosciences or eBioscience.

Multimers and monomers

K^b:OVA pentamers were used to stain OVA-specific CD8⁺ T cells (ProImmune) following T-cell magnetic enrichment by negative selection to remove CD19⁺ and CD11b⁺ cells. One test was used to stain 5 $\times$ 10⁶ cells resuspended at 50 $\times$ 10⁶ cells/ml.

H2-K^d- biotin monomers, H2-I-E^d-biotin monomers, H2-K^d or H2-I-E^d tetramers were obtained from the NIH tetramer Core Facility (Atlanta, GA). The peptides bound to H2-K^d and H2-I-E^d were SYIPSAEKI (Plasmodium berghei circumsporozoite peptide 252–260) (7) and SSIEFARL (herpes simplex virus glycoprotein B peptide 498 to 505) (8), respectively.

In some experiments, magnetic enrichment of tetramer positive B cells was performed before cell surface staining as previously described (9). For staining 5×10^6 cells, saturating (0.1 μ g) concentrations of each tetramer were used.

ELISpot

For detection of total IgG secreting B cells, plates (Millipore, Cat: MAIPSWU10) were coated with anti-mouse IgG F(ab)' specific antibody (Jackson ImmunoResearch, Cat: 115-005-072) overnight at 4 °C, then washed with PBS, and blocked with DMEM (10% FBS) 2 hours at 37°C. Cells were plated and cultured overnight, then washed and alkaline phosphatase conjugated anti-mouse IgG Fc specific antibody was added (Jackson ImmunoResearch, Cat: 115-055-071). After 1 hour at room temperature, plates were washed and developed with substrate BCIP/NPT (Sigma, Cat: B5655-25tab). For detecting H2-K^d specific IgG secreting plasma cells, plates were coated with anti-mouse IgG Fc-gamma specific antibody overnight at 4°C, washed and blocked. Cells (10^3 , 10^4 , and 10^5) were then plated and cultured overnight. Plates were washed and H2-K^d-biotin monomers were added. After one hour at room temperature, plates were washed and streptavidin-ALP (Mabtech, Cat:3310-10) was added. After one additional hour incubation at room temperature, plates were then washed and developed. The numbers of spots per well were enumerated using the ImmunoSpot Analyzer (CTL Analyzers LLC).

Donor-specific antibody (DSA) assay

For donor-specific antibody detection, serum was collected before and after transplantation and was diluted (1:50) and incubated with 1×10^6 BALB/c splenocytes resuspended at 20×10^6 cells/ml for 1 hour at 4°C. After washing twice, the cells were incubated with anti-IgG (Southern Biotech, Cat. # 1030-02) or anti-IgM (eBioscience, Cat. # 125790-82) and anti-B220 (BD Biosciences, Cat. # 561226) for 30 minutes. Mean fluorescence intensity (MFI) was measured by flow cytometry gating on B220^{lo} cells.

Results

CD4⁺ TCR-Tg T cell transfer leads to reduced intra-graft T cells

Adoptive transfer of antigen-specific TCR-Tg T cells is a tool most often used to follow monoclonal T cell responses and extrapolate conclusions to endogenous responses. In order to determine whether the adoptive transfer of TCR-Tg cells alters endogenous immune responses, a model of fully MHC-mismatched cardiac allograft transplantation with and without TCR-Tg cell transfer was examined. 1,000–250,000 (1K-250K) naïve CD4⁺ TCR75 T cells that recognize a BALB/c K^d peptide presented on I-A^b were transferred into C57BL/6 mice on the day of transplantation with a BALB/c heart (Figure 1A). The time of rejection was slightly accelerated in mice receiving 100K TCR75 cells (MST Day 6 vs Day 8 with no transfer, Figure 1B) and the majority of CD4⁺ cells present in the graft were TCR75 (79.4%±13.4%, Figure 1C). Unexpectedly, the TCR75 cell transfer led to a dose-dependent decrease in the total number of T cells within the rejected allografts, with the most striking reduction being in the number of endogenous CD8⁺ T cells (Figure 1D). Whereas graft-infiltrating CD8⁺ T cells vastly outnumbered CD4⁺ T cells in unmodified acute rejection (AR), the addition of 100K or more TCR75 cells not only reduced the

numbers of T cells in the graft but also shifted the ratio of CD8:CD4 T cells closer to 1:1 (Figure 1D).

We also examined the effects of an adoptive transfer of a second CD4⁺ TCR-Tg T cell, TEa, which recognizes an I-E^d peptide presented on I-A^b (Figure 1D). In contrast to the TCR75 cells, TEa cells comprised only a small proportion of the total CD4⁺ T cells within the allografts (4.4%±5.1%, Figure 1C). Despite this difference, TEa cells also mediated accelerated graft rejection that was characterized by reduced total numbers of graft-infiltrating T cells compared to rejection in the absence of TCR-Tg CD4⁺ T cells.

Accelerated graft rejection and reduction in total intra-graft T cells was specific to adoptive transfer of antigen-specific CD4⁺ TCR-Tg T cells, as transfer of similar numbers of naïve CD8⁺ TCR-Tg OT-I T cells into recipients of OVA-expressing BALB/c allografts or transfer of wild-type polyclonal CD4⁺ CD45.1⁺ T cells, which contain lower percentages of alloreactive T cells and a majority of T cells of irrelevant specificities, did not have these effects (Figure 1B,D). In fact, adoptive transfer of OT-I cells resulted in an increase in overall numbers of intra-graft T cells (Figure 1D).

One possibility for observing reduced numbers of T cells in Day 7-grafts from mice with CD4⁺ TCR-Tg T cell transfer was that we may have missed an earlier peak of T cell infiltration and T cells may have died or exited the graft by the time the mice were analyzed on Day 7. However, a time course analysis revealed that mice transferred with 100K TCR75 cells had few intra-graft T cells on days 3 and 5 post-transplantation, similarly to non-transferred animals (Figure 1E). Thus the addition of modest numbers of graft-specific CD4⁺ but not CD8⁺ TCR-Tg T cells profoundly limited the total accumulation of intra-graft CD4⁺ and CD8⁺ T cells.

CD4⁺ TCR-Tg T cells enhance graft-specific CD8⁺ T cell priming in the spleen

Another possibility for the observed reduction in intra-graft T cell numbers following CD4⁺ TCR-Tg T cell transfer was that the TCR-Tg cells were interfering with the priming of endogenous T cells in the spleen. To investigate this possibility, activated CD44^{hi} TCR-Tg and endogenous T cells were enumerated in the spleen 7–8 days following adoptive transfer of TCR75, TEa, OT-I or polyclonal T cells (Figure S1 and Figure 2). Rather than inhibiting T cell priming, transfer of CD4⁺ TCR-Tg T cells, but not OT-I or polyclonal cells, provided additional help to endogenous CD8⁺ T cells, with overall increased percentages and numbers of CD44^{hi} (Figure 2A) and interferon gamma (IFN γ)-producing (Figure 2B) CD8⁺ T cells in the spleens of those mice receiving TCR75 or TEa cells. There was no significant change in the percentages or numbers of regulatory T cells in the spleens of mice receiving CD4⁺ TCR-Tg T cells (Figure S2), suggesting that the CD4⁺ TCR-Tg T cells are not promoting stronger CD8⁺ T cell responses by affecting total numbers of Tregs or ratios of Tregs to T effector cells.

CD4⁺ helper T cells are so named for their ability to help other immune cells, including CD8 responses. To determine if TCR75 cell transfer could impact antigen-specific endogenous CD8⁺ T cells that recognize a different alloantigen than the K^d antigen recognized by the TCR75 cells, B6 mice were transplanted with BALB/c hearts expressing OVA so that

endogenous CD8⁺ T cell responses could be tracked using K^b:OVA multimers (Figure S3, 3A and 3B). Adoptive transfer of 100K TCR75 cells resulted in a 14-fold increase in the number of OVA-specific CD8⁺ T cells within the spleen (Figure 3B and 3C). These results indicate that TCR75 transfer can alter endogenous CD8⁺ T cell responses that do not require the recognition of the same alloantigen. However, despite the increased accumulation of splenic K^b:OVA-reactive cells in the spleen, the TCR75 transfer resulted in a log-decrease in the numbers of intra-graft K^b:OVA-reactive CD8⁺ T cells (Figure 3C). Thus TCR75 transfer enhanced non-cognate endogenous allospecific CD8⁺ T cell accumulation in the spleen but inhibited their accumulation in the graft in a similar manner to what was observed for polyclonal CD8⁺ T cells.

CD4⁺ TCR-Tg T cells enhance allograft-specific B cell responses

In addition to helping CD8⁺ T cell responses, CD4⁺ T cells are also known to provide B cell help. To determine whether the transferred CD4⁺ TCR75 cells were similarly providing help to alloreactive B cells, splenocytes were first stained with CXCR5 and PD-1, which are markers of T follicular helper cells (Tfh cells). A high proportion of transferred TCR75 cells differentiated into Tfh cells while the number of endogenous Tfh cells remained similar in transferred versus non-transferred mice (Figure S4); thus, in mice receiving TCR75 cells, the total numbers of Tfh cells were effectively doubled (Figure 4A). This finding correlated with an expansion of allospecific B cells that recognized K^d, which reflects their ability to engage in cognate interaction with the TCR75 cells (Figure 4B). The K^d-specific B cells were not enriched in cells expressing germinal center markers (GL-7⁺, Fas⁺, data not shown). Instead, they had already begun to differentiate into plasmablasts that downregulate B220 (Figure 4C) and CD19 (Figure S5) by the time of acute rejection. Indeed, as measured by ELISpot, TCR75 transfer increased antibody-producing splenocytes at day 7 post-transplantation (Figure 4D) and serum IgM and IgG alloantibodies as early as 6 days post-transplantation (Figure 4E).

We also quantified the numbers of Class II (I-E^d)-specific B cells, which are not able to engage in cognate interactions with TCR75 cells. While the total numbers of I-E^d-specific B cells were not significantly increased, the proportion of these B cells that downregulated B220 was significantly augmented (Figure 4C). In addition, the ELISpot revealed many more total antibodies produced than those specific for K^d (Figure 4D), illustrating that TCR75 transfer enhances the differentiation of alloreactive B cells that can engage in both cognate and non-cognate interactions with TCR75 cells.

To further illustrate the potency of the help provided by transferred CD4⁺ TCR-Tg T cells, we transplanted the quasi monoclonal MD4 BCR-Tg mice with or without transferred TCR75 cells. As 90% of B cells in MD4 mice are specific for Hen Egg Lysozyme (HEL) (10), these animals provide a model in which most B cells are not capable of producing alloantibodies. Remarkably, transfer of 100K TCR75 cells was able to drive allospecific IgG production by day 6 in these mice (Figure S6A). These observations underscore the potency of transferred TCR75 cells at providing B cell help, even in the face of a dramatically reduced alloreactive B cell repertoire. Notably, TCR75 transfer into MD4 hosts still resulted in reduced intra-graft T cell infiltration (Figure S6B).

Biopsies of patients undergoing acute antibody-mediated rejection often contain minimal cellular infiltrates (11). Because of the reduced intra-graft cellular infiltrate upon CD4⁺ TCR-Tg T cell transfer, we tested the possibility that the allografts in the presence of TCR75 underwent antibody-mediated instead of T cell-mediated rejection. To test whether alloantibodies and their downstream effects were inhibiting intra-graft T cell accumulation in animals that had received CD4⁺ TCR-Tg cell transfer we used μ MT^{-/-} mice, which lack membrane-bound IgM and mature B cells. μ MT^{-/-} mice did not make allo-IgM or allo-IgG upon transplantation and transfer of 100K TCR75 cells (Figure S6C). However, accelerated graft rejection (Figure 4F) and a reduction of T cells within the allografts were still observed (Figure 4G). Collectively these observations support the conclusion that early production of alloantibodies was not required for the reduction of intra-graft T cell accumulation following CD4⁺ TCR-Tg T cell transfer.

The transfer of CD4⁺ TCR-Tg T cells alters intra-graft accumulation of innate immune cells

To determine if CD4⁺ TCR-Tg T cell transfer had additional consequences on the endogenous alloresponse and gain insight into the mechanism of graft rejection, we analyzed the phenotype of other intra-graft leukocytes. TCR75 cell transfer resulted in a dose-dependent decrease in B cells and CD11c⁺ dendritic cells in the grafts (Figure 5A), and a striking increase in neutrophils (Figure 5B). Intra-graft CD11c⁺ cells were also decreased and neutrophils increased when TEa cells were transferred (data not shown). This reduction in graft-infiltrating antigen-presenting cells was unique to the CD4⁺ TCR-Tg T cell transfer, as OT-I transfer had no effect on B cell accumulation, and increased CD11c⁺ cells within the allografts (Figure S7A), although similarly to the CD4⁺ TCR-Tg T cell transfer, OT-I transfer also resulted in increased neutrophils in the graft (Figure S7A). Antigen specificity was required for T cell transfer to have an effect on accumulation of endogenous immune cell populations, as transfer of 250K polyclonal CD4s plus 200K polyclonal CD8s had no effect on B cell, CD11c⁺ cell or neutrophil accumulation compared to mice without transfer (Figure S7B).

As the total numbers of graft-infiltrating T cells was lowest in mice with TCR75 transfer and highest in mice with OT-I transfer, and this trend was recapitulated with intra-graft CD11c⁺ cells, we tested whether there was a correlation between the two factors. To this end, data from 38 animals were combined from each of the groups of non-transfer, TCR75, TEa, OT-I and polyclonal T cell transfer. There was a robust positive correlation between the numbers of CD11c⁺ cells and the total number of intra-graft T cells (Figure 5C, Pearson $r=0.66$, $p<0.0001$), suggesting that transfer of CD4 or CD8 TCR-Tg cells may affect the numbers of intra-graft CD11c⁺ cells, and these intra-graft CD11c⁺ cells may in turn affect the total numbers of intra-graft T cells, as has been described in tumor settings (12).

Discussion

TCR-Tg T cells are useful tools for following antigen-specific immune responses *in vivo* but they must be used cautiously. Using infectious disease models, it has been previously shown that OVA-specific OT-I cells can effectively compete for antigen with endogenous OVA-reactive CD8⁺ T cells, such that only transfer of less than 500 TCR-Tg OT-I cells can

preserve the endogenous T cell response to the same antigen (5). Our data is consistent with this conclusion as few endogenous OVA-specific T cells were detectable in the graft following OT-I transfer (K^b :OVA⁺CD45.1⁺, Figure 1C). Badovinac et al as well as others reported that CD8 memory T cell differentiation kinetics depended on initial precursor frequency (5,13,14). Ford and colleagues have demonstrated different levels of resistance of TCR-Tg T cells to costimulation blockade based on their initial seeded frequency in a skin transplant model (2). For CD4⁺ TCR-Tg cells, Marshall et al compared the magnitude of the response of CD4⁺ SMARTA T cells with that of endogenous T cells reactive to the same LCMV antigen, but in separate mice (15). This study concluded that SMARTA T cells were representative of the endogenous response but no analysis of endogenous responses in the mice that received SMARTA T cells, compared to those that did not receive SMARTA cells, was shown (15).

Our study shows, in contrast, that the addition of naïve CD4⁺ TCR-Tg T cells not only likely competes with T cells recognizing the same antigen but also alters the response of CD4⁺ and CD8⁺ T cells recognizing other donor-specific antigens following allograft transplantation. The numbers and types of non-T cell subsets able to accumulate within the allografts were also altered by the addition of modest numbers of CD4⁺ TCR-Tg T cells, further demonstrating a significant reshaping of the endogenous alloresponse by the transferred graft-reactive T cells. Of the hematopoietic cell populations that were altered in the graft, the reduction in intra-graft T cells correlated most strongly with a reduction in the number of intra-graft CD11c⁺ cells. This result is interesting in the context of recent data from Spranger et al who showed in a melanoma model that the presence of CD11c⁺ cells positively correlated with intra-tumoral T cells, and that the addition of dendritic cells intra-tumorally allowed for increased T cell accumulation (12). Antigen-specific CD4⁺ T cells have been shown to have the ability to kill antigen-presenting cells (APCs) (16,17), and APCs have been shown to be particularly sensitive to CD4-mediated killing in the presence of high numbers of CD4⁺ TCR-Tg T cells (18). Therefore, a possible explanation for the reduction in CD11c⁺ cells observed in allografts with TCR75 transfer is that the TCR-Tg cells may have killed some of the APCs. The reduced number of APCs could then lead to a reduction in the chemokines produced by APCs that attract T cells or retain them within the allograft.

The reshaped endogenous immune response observed with TCR75 cell transfer was also observed with transfer of T cells from a second CD4⁺ TCR-Tg mouse, TEa, although the TEa cells infiltrated the grafts at a lower frequency (Figure 1) and accumulated in the spleen at lower numbers (Figure S1). Differences in intra-graft accumulation of these two types of TCR-Tg T cells may be due to the respective antigens recognized by each TCR (K^d for the TCR75 cells which is expressed by all cells in the allograft versus I-E for TEa cells which is only expressed on professional antigen-presenting cells and activated endothelial cells within the graft), or to a difference in the TCR affinity of each monoclonal population. Despite these differences, our data suggest that transfer of TEa cells is also associated with a decrease in CD11c⁺ cells, correlating with a reduction in the accumulation of endogenous allograft-specific T cells in the allograft.

Many groups utilizing TCR-Tg T cells in transplantation studies have transferred much higher numbers of T cells than those used here, up to 2×10^7 T cells (2,19–21). In this study, as few as 10^4 TCR75 cells had profound effects on the endogenous immune response, with elevated alloantibody responses detected with as few as 10^3 cells (data not shown), and with greater perturbations with increasing numbers of transferred TCR-Tg T cells. In light of these observations, some past studies should be revisited and perhaps re-interpreted. For example, if a new immunosuppressive agent were to be tested in the mice used in this study it would not only have to overcome the effects of the adoptively transferred $CD4^+$ T cells specific for the graft, but also would have to counter an enhanced (in magnitude and kinetics) endogenous $CD8$ and alloantibody response. Thus, the efficacy of the immunosuppressive agent may be significantly underestimated. Future investigations using these cells should limit the numbers of TCR-Tg cells used for transfer so studies may more accurately reflect endogenous precursor frequencies.

Interestingly the naïve $CD4^+$ TCR-Tg T cell transfer was able to promote both cognate and non-cognate allospecific B cell responses. This result supports published data showing that B cells are directly able to receive help from non-cognate $CD4^+$ T cells provided these B cells express MHC-II to present the antigen and have acquired the non-cognate antigen from the same cell that expresses the cognate antigen (22). Our data also show that transfer of $CD4^+$ TCR-Tg T cells promotes a non-cognate alloreactive $CD8^+$ T cell response, which invokes an indirect and/or semi-direct model of help. This could occur when the $CD4^+$ T cell activates APCs that present both alloantigens, such that the APCs, in turn, can stimulate the $CD8^+$ T cells that recognize a distinct alloantigen from that recognized by the $CD4^+$ TCR-Tg T cells.

Overall, the data give insight into the “cooperativity” of rejection first described by Bucy and colleagues, who when adoptively transferring TCR75 cells into C57BL/6 recipients of B6.K^d hearts noted a non-linear relationship between the number of cells transferred and the kinetics of rejection (3). This is likely due to reaching a threshold number of $CD4^+$ T cells that not only facilitate rejection themselves but also coordinate the rejection event by promoting more $CD8^+$ T cell responses in the spleen and a strong alloantibody response. Indeed, in B cell-competent mice, $CD4^+$ but not $CD8^+$ T cells have been shown to be essential for cardiac allograft rejection (23). However, in B cell-deficient mice depleted of $CD8^+$ T cells, the remaining naïve $CD4^+$ T cells were incapable of rejecting a cardiac allograft (24), suggesting that $CD4$ help to either $CD8^+$ T cells or B cells is necessary to elicit graft rejection. In contrast, memory $CD4^+$ T cells might lead to allograft rejection by other mechanisms, as they could still trigger rejection in B cell-deficient mice depleted of $CD8^+$ T cells (24). Our data reveal increasing the numbers of naïve alloreactive $CD4^+$ T cells provides enhanced help to $CD8^+$ T cells and allospecific B cells but it also leads to an overall reduction in intragraft T cells as well as $CD11c^+$ cells. The mechanism of rejection is most likely altered by the presence of these increased numbers of $CD4^+$ T cells, as graft rejection ensues without ever achieving high numbers of T cells within the graft and can still be accelerated without alloantibodies as the results of the $\mu MT^{-/-}$ experiments showed. It is possible that the transferred $CD4^+$ TCR-Tg T cells provide help to allospecific $CD8^+$ T cells, which in turn may destroy donor endothelial cells, causing graft rejection before many T cells infiltrate the graft.

Our data showing that transfer of naïve TCR75 cells which results in reduced accumulation of T cells, B cells and dendritic cells in the allograft contrasts with data obtained by Martin-Orozco and colleagues in a tumor model. In this B16-OVA melanoma model, transfer of activated, Th17-skewed CD4⁺ TCR-Tg OT-II T cells enhanced endogenous CD4, CD8, antigen-presenting cell and neutrophil accumulation into the tumor (25). Whether the differences are due to the tumor microenvironment or to the TCR-Tg T cells being pre-activated rather than naïve remains to be established.

CD8⁺ T cell transfer also perturbed endogenous alloimmunity, albeit in different ways than the CD4⁺ T cell transfer. CD8⁺ T cell transfer led to an overall increase in the numbers of graft-infiltrating T cells, CD11c⁺ and neutrophils, though it did not accelerate graft rejection. Taken with CD4⁺ T cell transfer data, the alterations in endogenous alloimmunity following transfer of CD8⁺ T cells provides evidence that there are unique effects of distinct adoptively transferred populations on endogenous alloimmune responses.

Assessing the effects of adoptive transfer of antigen-specific T cells in a clinical setting should be the subject of future investigations as recently completed and ongoing clinical trials are evaluating the efficacy of transferring very large numbers of expanded antigen-specific T cells in settings of tumor and of viral infection, (for example NCT00393029, NCT02210104, NCT00880789, NCT00110578) (26). To date, endogenous immune responses have not been monitored in these patients and it is not known if the adoptive cell therapy would have unintended long-term consequences on the endogenous immune response against the tumor or virus being targeted, or against a concurrent infection. Clinical trials are also ongoing in transplantation, transferring expanded antigen-specific Tregs to prolong allograft survival, anti-tumor T cells to combat post-transplant lymphoproliferative disorder (PTLD) or viral-specific T cells to control infection (NCT02371434, NCT00063648, NCT00880789). Supporting our results, one recent preclinical study using adoptive Treg therapy in a non-human primate model of heart transplantation revealed that transfer of Tregs into a lymphopenic host can result in enhanced alloantibody responses and T cell memory, as the transferred cells provided help to endogenous alloreactive cells (27). Whereas our study assessed the consequences of transferring naïve T cells, many clinical trials use transfer of activated T cells, which may alter the endogenous immune response differently. In addition, humans have a higher proportion of endogenous memory T cells than laboratory mice, which may react differently than naïve T cells to the adoptive cell therapy. The consequences of T cell transfers on endogenous immune responses should be investigated in human clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Jessalynn Holman for breeding and genotyping all of the animals and the University of Chicago Flow Cytometry Facility for help with the instruments. We would like to thank the NIH tetramer Core Facility for providing pMHC tetramers and monomers. M.L.M. was funded by AHA predoctoral fellowships (13PRE14550022 and 15PRE22180007), a Cardiovascular Pathophysiology and Biochemistry Training Grant (T32

HL07237), and an HHMI Med-into-Grad Program training grant (56006772). The work was also supported by NIAID P01AI-97113 to A.S.C. and M.-L.A.

Abbreviations

ANOVA	analysis of variance
APC	antigen-presenting cell
AR	acute rejection
DMEM	Dulbecco modified Eagle medium
DSA	Donor specific antibody
HEL	Hen Egg Lysozyme
HBSS	Hanks balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N'-2- ethanesulfonic acid
IFNγ	Interferon gamma
MFI	Mean fluorescence intensity
n	number in group
NA	not applicable
ns	not significant
P	probability
PBS	phosphate-buffered saline
PMA	phorbol myristate acetate
PTLD	post-transplant lymphoproliferative disorder
Th	T helper [cell]
OVA	ovalbumin
TCR-Tg	T cell receptor transgenic
Tfh	T follicular helper

References

1. Wirth TC, Pham N-LL, Harty JT, Badovinac VP. High initial frequency of TCR-transgenic CD8 T cells alters inflammation and pathogen clearance without affecting memory T cell function. *Mol Immunol*. 2009 Nov; 47(1):71–78. [PubMed: 19195704]
2. Ford ML, Koehn BH, Wagener ME, Jiang W, Gangappa S, Pearson TC, et al. Antigen-specific precursor frequency impacts T cell proliferation, differentiation, and requirement for costimulation. *J Exp Med*. 2007 Feb 19; 204(2):299–309. [PubMed: 17261633]

3. Honjo K, Yan Xu X, Kapp JA, Bucy RP. Evidence for cooperativity in the rejection of cardiac grafts mediated by CD4 TCR Tg T cells specific for a defined allopeptide. *Am J Transplant*. 2004 Nov; 4(11):1762–1768. [PubMed: 15476474]
4. Jensen SM, Twitty CG, Maston LD, Antony PA, Lim M, Hu H-M, et al. Increased Frequency of Suppressive Regulatory T Cells and T-cell Mediated Antigen Loss Results in Murine Melanoma Recurrence. *J Immunol*. 2012 Jul 15; 189(2):767–776. [PubMed: 22723522]
5. Badovinac VP, Haring JS, Harty JT. Initial T cell receptor transgenic cell precursor frequency dictates critical aspects of the CD8(+) T cell response to infection. *Immunity*. 2007 Jun; 26(6):827–841. [PubMed: 17555991]
6. Corry RJ, Winn HJ, Russell PS. Primarily vascularized allografts of hearts in mice. The role of H-2D, H-2K, and non-H-2 antigens in rejection. *Transplantation*. 1973 Oct; 16(4):343–350. [PubMed: 4583148]
7. Kessler B, Michielin O, Blanchard CL, Apostolou I, Delarbre C, Gachelin G, et al. T cell recognition of hapten. Anatomy of T cell receptor binding of a H-2kd-associated photoreactive peptide derivative. *J Biol Chem*. 1999 Feb 5; 274(6):3622–3631. [PubMed: 9920911]
8. Blaney JE, Nobusawa E, Brehm MA, Bonneau RH, Mylin LM, Fu TM, et al. Immunization with a single major histocompatibility complex class I-restricted cytotoxic T-lymphocyte recognition epitope of herpes simplex virus type 2 confers protective immunity. *J Virol*. 1998 Dec; 72(12):9567–9574. [PubMed: 9811690]
9. Chen J, Wang Q, Yin D, Vu V, Sciammas R, Chong AS. Cutting Edge: CTLA-4Ig Inhibits Memory B Cell Responses and Promotes Allograft Survival in Sensitized Recipients. *J Immunol*. 2015 Nov 1; 195(9):4069–4073. [PubMed: 26416270]
10. Chen J, Yin H, Xu J, Wang Q, Edelblum KL, Sciammas R, et al. Reversing endogenous alloreactive B cell GC responses with anti-CD154 or CTLA-4Ig. *Am J Transplant*. 2013 Sep; 13(9):2280–2292. [PubMed: 23855587]
11. Berry GJ, Burke MM, Andersen C, Bruneval P, Fedrigo M, Fishbein MC, et al. The 2013 International Society for Heart and Lung Transplantation Working Formulation for the standardization of nomenclature in the pathologic diagnosis of antibody-mediated rejection in heart transplantation. *J Heart Lung Transplant*. 2013 Dec; 32(12):1147–1162. [PubMed: 24263017]
12. Spranger S, Bao R, Gajewski TF. Melanoma-intrinsic β -catenin signalling prevents anti-tumour immunity. *Nature*. 2015 Jul 9; 523(7559):231–235. [PubMed: 25970248]
13. Marzo AL, Klonowski KD, Le Bon A, Borrow P, Tough DF, Lefrançois L. Initial T cell frequency dictates memory CD8+ T cell lineage commitment. *Nat Immunol*. 2005 Aug; 6(8):793–799. [PubMed: 16025119]
14. Hataye J, Moon JJ, Khoruts A, Reilly C, Jenkins MK. Naive and memory CD4+ T cell survival controlled by clonal abundance. *Science*. 2006 Apr 7; 312(5770):114–116. [PubMed: 16513943]
15. Marshall HD, Chande A, Jung YW, Meng H, Poholek AC, Parish IA, et al. Differential expression of Ly6C and T-bet distinguish effector and memory Th1 CD4(+) cell properties during viral infection. *Immunity*. 2011 Oct 28; 35(4):633–646. [PubMed: 22018471]
16. Umeshappa CS, Huang H, Xie Y, Wei Y, Mulligan SJ, Deng Y, et al. CD4+ Th-APC with acquired peptide/MHC class I and II complexes stimulate type 1 helper CD4+ and central memory CD8+ T cell responses. *J Immunol*. 2009 Jan 1; 182(1):193–206. [PubMed: 19109150]
17. Grogg D, Hahn S, Erb P. CD4+ T cell-mediated killing of major histocompatibility complex class II-positive antigen-presenting cells (APC). III. CD4+ cytotoxic T cells induce apoptosis of APC. *Eur J Immunol*. 1992 Jan; 22(1):267–272. [PubMed: 1346113]
18. Ingulli E, Mondino A, Khoruts A, Jenkins MK. In Vivo Detection of Dendritic Cell Antigen Presentation to CD4+ T Cells. *J Exp Med*. 1997 Jun 16; 185(12):2133–2141. [PubMed: 9182685]
19. Burrell BE, Bromberg JS. Fates of CD4+ T Cells in a Tolerant Environment Depend on Timing and Place of Antigen Exposure. *Am J Transplant*. 2012 Mar; 12(3):576–589. [PubMed: 22176785]
20. Conlon TM, Saeb-Parsy K, Cole JL, Motallebzadeh R, Qureshi MS, Rehakova S, et al. Germinal center alloantibody responses are mediated exclusively by indirect-pathway CD4 T follicular helper cells. *J Immunol*. 2012 Mar 15; 188(6):2643–2652. [PubMed: 22323543]

21. Diamond AS, Gill RG. An essential contribution by IFN-gamma to CD8+ T cell-mediated rejection of pancreatic islet allografts. *J Immunol.* 2000; 165(1):247–255. [PubMed: 10861058]
22. Conlon TM, Cole JL, Motallebzadeh R, Harper I, Callaghan CJ, Bolton EM, et al. Unlinked memory helper responses promote long-lasting humoral alloimmunity. *J Immunol.* 2012 Dec 15; 189(12):5703–5712. [PubMed: 23162131]
23. Krieger NR, Yin DP, Fathman CG. CD4+ but not CD8+ cells are essential for allorejection. *JExpMed.* 1996; 184:2013–2018.
24. Nozaki T, Rosenblum JM, Ishii D, Tanabe K, Fairchild RL. CD4 T cell-mediated rejection of cardiac allografts in B cell-deficient mice. *J Immunol.* 2008 Oct 15; 181(8):5257–5263. [PubMed: 18832680]
25. Martin-Orozco N, Muranski P, Chung Y, Yang XO, Yamazaki T, Lu S, et al. T helper 17 cells promote cytotoxic T cell activation in tumor immunity. *Immunity.* 2009 Nov 20; 31(5):787–798. [PubMed: 19879162]
26. Aranda F, Buqué A, Bloy N, Castoldi F, Eggermont A, Cremer I, et al. Trial Watch: Adoptive cell transfer for oncological indications. *Oncoimmunology.* 2015 Nov.4(11):e1046673. [PubMed: 26451319]
27. Ezzelarab MB, Zhang H, Guo H, Lu L, Zahorchak AF, Wiseman RW, et al. Regulatory T Cell Infusion Can Enhance Memory T Cell and Alloantibody Responses in Lymphodepleted Nonhuman Primate Heart Allograft Recipients. *Am J Transplant.* 2015 Dec 23.

Figure 1. Adoptive transfer of CD4⁺ TCR-Tg T cells prevented accumulation of transferred and endogenous CD4⁺ and CD8⁺ T cells

A. Experiment design. B. Graft survival following transplantation without T cell transfer ($\emptyset$, n=14) or with 10^5 TCR75 (100K, n=7), 2.5×10^5 TEa (250K, n=6), 10^5 OT-I (100K, n=6), or 2.5×10^5 CD4⁺ plus 2×10^5 CD8⁺ polyclonal T cells (poly, n=6). C. Representative flow cytometry plots of intra-graft T cells of mice receiving TCR75 (10K–250K cells transferred, n=16), TEa (250K cells transferred, n=6), OT-I (100K cells transferred, n=6) and polyclonal T cells (250K CD4⁺ + 200K CD8⁺ cells transferred, n=6). Numbers are the mean percentage of transferred cells out of CD4⁺ or CD8⁺ graft-infiltrating T cells $\pm$ SD. D. Numbers of

endogenous CD4⁺, TCR-Tg, and CD8⁺ cells infiltrating the grafts 6–8 days post-transplantation, and ratio of CD8:CD4 T cells; Ø(no transfer, n=14), TCR75: 1K (n=6) 10K (n=3) 100K (n=6) 250K (n=7), TEa: 250K (n=6), OT-I: 100K (n=6), polyclonal (250K CD4⁺ + 200K CD8⁺ cells transferred, n=6). E. Numbers of total CD4⁺ and CD8⁺ T cells infiltrating the grafts on days 3, 5, or 7–8 post-transplantation; Ø (no transfer, n=3,3,14), +100K TCR75: (n=3,3,6). Data from Day 7–8 is the same as in 1D. Results are pooled from 2–10 independent experiments. Survival data was analyzed by log-rank test, and mean values were compared using one-way analysis of variance (ANOVA) (D) or two-way ANOVA (E) with Bonferroni correction for pairwise comparisons, ns=not significant, *p<0.05, **<p<0.01, ***p<0.001

Figure 2. Adoptive transfer of CD4⁺ TCR-Tg T cells augmented endogenous CD8⁺ T cell responses in the spleen

Percentages and numbers of CD44^{hi} (A) or IFN γ ⁺ (B) endogenous CD8⁺ T cells in the spleens of mice receiving no transfer of cells (n=22), adoptive transfer of TCR75 (10K–250K cells, n=12–13), TEa (250K cells, n=5–6), OT-I (100K cells, n=6) or polyclonal (poly, 250K CD4⁺ + 200K CD8⁺ cells, n=6), and did (acute rejection, AR, with or without transfer) or did not (naïve) receive a heart transplant analyzed on day 7–8 post-transfer or post-transplantation. Mean values were compared using one-way analysis of variance (ANOVA) with Bonferroni correction for pairwise comparisons, ns=not significant, *p<0.05, ***p<0.001 for TCR75 groups #p<0.05 for TEa groups.

Figure 3. TCR75 cell transfer affected endogenous CD8⁺ T cell responses even when they recognized distinct antigens

A. C57BL/6 mice were transplanted with OVA-expressing BALB/c × B6 F1 hearts and a subset of mice were adoptively transferred with 100K TCR75 cells. B. Percentage of K^b:OVA⁺ cells in the spleen ± SD (n=5–6 per group). C. Numbers of endogenous K^b:OVA⁺ cells identified in the spleen (n=5–6 mice per group) and graft (n=6 per group) 8–9 days post transplantation. All results pooled from 2 independent experiments. Mean values were compared using Student's t test, *p<0.05, **p<0.01.

Figure 4. CD4⁺ TCR-Tg T cell transfer led to increased cognate and non-cognate alloantibody responses

A. Percentages and total numbers of Tfh cells (PD-1^{hi}, CXCR5^{hi}) B. Numbers of K^d-specific (MHC I) and I-E^d specific (MHC II) B cells in the spleens of naïve mice or mice undergoing acute rejection with or without TCR75 transfer. C. Percentages of B220^{lo} cells amongst MHC I- or MHC II-specific B cells. For A–C, n=4–6 per group pooled from two independent experiments. D. ELISpot of antibody-producing cells that made alloantibodies (total IgG) or to specifically to K^d. 10⁴ cells were plated per well. Data are representative of two experiments of triplicate wells for each of 2–3 mice per group. E. Donor-specific

antibodies were measured in the serum one day prior to transplantation and on days 4 and 6 post-transplantation. n=3 mice per group, representative of 2 independent experiments. F and G. Survival of hearts post-transplantation in wild-type (WT) or $\mu\text{MT}^{-/-}$ mice with or without 10^5 TCR75 cells (+100K) (F) and numbers of endogenous CD4, CD8, and TCR-Tg cells infiltrating the grafts 6–8 days post transplantation (G); $\emptyset$ (no transfer, n=14), 100K TCR75 (n=6) $\mu\text{MT}^{-/-}$ (n=6), $\mu\text{MT}^{-/-}$ + 100K TCR75 (n=6). For F & G, data from WT and WT+100K TCR75 are the same as those shown in Figure 1, and data are pooled from two experiments. Mean values were compared using Student's t-test or one-way ANOVA with Bonferroni correction for pairwise comparisons where appropriate **p<0.01, ***p<0.001.

Figure 5. Transfer of TCR75 cells altered the numbers of non-T cell populations in the graft
 C57BL/6 mice were transplanted with BALB/c heart grafts. Six to eight days post-transplantation intra-graft antigen-presenting cells and neutrophils were enumerated by flow cytometry. **A.** Numbers of intra-graft B cells and CD11c⁺ cells in mice with no transfer (Ø, n=14), 10K TCR75 cells (n=3) or 100K TCR75 cells (n=6). **B.** Representative flow cytometry plots of mean percentages of graft-infiltrating neutrophils (Gr-1^{hi}, CD11b^{hi}) ± SD after gating on CD45⁺TCRβ⁻CD19⁻ cells and their total numbers in mice with no transfer (n= 17), 10K TCR75 cells (n=3) or 100K TCR75 cells (n=6). For **A** and **B**, mean values

were compared using one-way ANOVA with Bonferroni correction for pairwise comparisons * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. C. Numbers of intra-graft CD11c⁺ cells versus total T cells are positively correlated, Pearson $r = 0.66$, $p < 0.0001$. Results are pooled from 2–10 experiments.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript